Aldehydes in Exhaled Breath Condensate of Patients with Chronic Obstructive Pulmonary Disease

Massimo Corradi, Israel Rubinstein, Roberta Andreoli, Paola Manini, Andrea Caglieri, Diana Poli, Rossella Alinovi, and Antonio Mutti

National Institute of Occupational Safety and Prevention Research Center, and Laboratory of Industrial Toxicology, Department of Clinical Medicine, Nephrology and Health Sciences, University of Parma, Parma, Italy; Department of Respiratory and Critical Care Medicine, College of Medicine, and Department of Pharmaceutics and Pharmacodynamics, College of Pharmacy, University of Illinois at Chicago; and Veterans Affairs Chicago Health Care System, West Side Division, Chicago, Illinois

The aims of the present study were (*1*) to evaluate whether individual aldehydes resulting from lipid peroxidation can be measured in exhaled breath condensate, (*2*) to assess the influence of sampling procedures on aldehyde concentrations, and (*3*) to compare aldehyde levels of patients with stable, moderate to severe, chronic obstructive pulmonary disease with those of smoking and nonsmoking control subjects. Aldehydes (malondialdehyde, hexanal, heptanal, and nonanal) were measured by liquid chromatography-tandem mass spectrometry in all samples and overlapping results were obtained by different sampling procedures. Malondialdehyde (57.2 ± 2.4 nmol/L), hexanal (63.5 ± 4.4 nmol/L), and heptanal (26.6 ± 3.9 nmol/L) were increased in patients as compared with nonsmoking control subjects (17.7 ± 5.5 nmol/L, $p < 0.0001$; 14.2 ± 3.5 nmol/L, $p = 0.004$; and 18.7 ± 0.9 nmol/L, $p = 0.002$, respectively). Only malondialdehyde was increased in patients compared with smoking control subjects (35.6 ± 4.0 nmol/L, $p = 0.0007$). In conclusion, different classes of aldehydes were identified in exhaled breath condensate of humans. Whereas all aldehydes but nonanal were lower in control subjects as compared with other groups, only malondialdehyde distinguished smoking control subjects from patients with chronic obstructive pulmonary disease and could be envisaged as a biomarker potentially useful to monitor the disease and its response to therapy.

Keywords: aldehydes; biomarkers; COPD; exhaled breath condensate; reproducibility

Oxidative stress is enhanced in patients with chronic obstructive pulmonary disease (COPD), because of free radicals in tobacco smoke and reactive oxygen species (ROS) produced by activated inflammatory cells (1, 2).

Reliable and noninvasive methods to assess oxidative stress might be useful to identify smokers at a greater risk of developing COPD and might shed new light on its natural history, thus providing a rationale for the assessment of conventional therapies (3) and for the development of novel drugs (4).

Exhaled breath condensate (EBC) obtained by cooling exhaled air during spontaneous breathing is a biological matrix that could provide a direct assessment of pulmonary pathobiology (5, 6). Biomarkers of oxidative stress are increased in the EBC of patients with COPD (7–10). However, the validity of EBC seems to be questionable because of analytical problems associated with the use of immunochemical and colorimetric assays affected by poor sensitivity, specificity, and selectivity. Moreover, there is a need to assess the influence of sampling procedures on EBC biomarkers, before their clinical application (11).

Among the mechanisms of ROS damage, lipid peroxidation is probably the most extensively investigated process. Oxidation of cell membrane phospholipids produces a chain reaction, the targets of which are the polyunsaturated fatty acids, and results in the formation of unstable lipid hydroperoxides and of secondary carbonyl compounds, such as aldehydic products (12). Among these, malondialdehyde (MDA) is the most frequently reported in the literature. MDA can be measured as a thiobarbituric acid-reactive substance in EBC (13), which is increased in patients with clinically stable COPD (14). This colorimetric assay has been criticized because of its lack of specificity and because thiobarbituric acid-reactive substances are formed during sample preparation (15). Therefore, more specific analytical methods must be used to provide evidence of validity of such biomarkers in EBC. Besides MDA, which is generated mainly by arachidonic acid and docosahexenoic acid, other aldehydes are produced during lipid peroxidation: α,β-unsaturated aldehydes, namely 4-hydroxynonenal and 4-hydroxyhexenal, are formed by peroxidation of ω-6 (e.g., arachidonic and linoleic acid) and ω-3 polyunsaturated fatty acids (e.g., oleic acid); saturated aldehydes (hexanal, heptanal, and nonanal) are known to be breakdown products of oxidized linoleic and arachidonic, palmitoleic, and oleic acid, respectively (15, 16).

In this study, we set up a new analytical method for the simultaneous determination of different classes of aldehydes in EBC, using liquid chromatography–tandem mass spectrometry, which is considered to be a reference technique to analyze organic compounds in aqueous matrices (17). The aim of the study was (*1*) to apply liquid chromatography–tandem mass spectrometry for the identification and quantification of different classes of aldehydes in EBC, (*2*) to evaluate the influence of EBC-sampling procedures on EBC aldehyde levels, and (*3*) to verify whether patients with COPD exhale more aldehydes than do smoking and nonsmoking control subjects.

METHODS

Subjects

Twenty patients with stable, moderate to severe COPD (18 men and 2 women; mean age, 63.3 ± 14.6 years), 12 smoking control subjects (9 men and 3 women; mean age, 41.7 ± 13.9 years), and 20 nonsmoking control subjects (17 men and 3 women; mean age, 55.0 ± 6.0 years) were enrolled. All the patients with COPD met the Global Initiative for Chronic Obstructive Lung Disease criteria for the diagnosis of COPD

(Received in original form October 31, 2002; accepted in final form January 8, 2003)

Supported in part by a VA Merit Review Grant and by grant 1R01 HL72323–01 from the NHLBI. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NHLBI or NIH.

Correspondence and requests for reprints should be addressed to Antonio Mutti, M.D., Laboratory of Industrial Toxicology, Department of Clinical Medicine, Nephrology and Health Sciences, Via Gramsci 14, University of Parma, 43100 Parma, Italy. E-mail: antonio.mutti@unipr.it

Am J Respir Crit Care Med Vol 167. pp 1380–1386, 2003
Originally Published in Press as DOI: 10.1164/rccm.200210-1253OC on January 9, 2003
Internet address: www.atsjournals.org

(18). Patients with COPD (baseline FEV_1, 52.1 ± 20.1% of predicted) had fixed airflow obstruction, which was defined as postbronchodilator $FEV_1/FVC < 70\%$. None was receiving inhaled steroids for 6 weeks before testing, nor was receiving intravenous/oral corticosteroids for at least 6 months before the EBC collection. Seven were current smokers (51.4 ± 41.2 pack-years) and 13 were ex-smokers (31.6 ± 18.3 pack-years). Asymptomatic smoking control subjects (FEV_1, 100.6 ± 22.0% of predicted; smoking history, 20.0 ± 21.2 pack-years) were recruited. Control subjects were defined as self-reported life-long nonsmoking individuals without a significant history of respiratory diseases and with normal spirometry, the mean baseline FEV_1 being 96.2 ± 9.7% of predicted.

The study has been approved by University of Illinois at Chicago Institutional Review Board.

Exhaled Breath Condensate Collection

EBC was collected with a simple apparatus described previously (5, 19). Briefly, subjects sitting comfortably in the laboratory performed repeated slow vital capacities into a Tygon tube (Nalgene 890 FEP tubing; Nalge Nunc International, Naperville, IL) immersed in thawing ice. Subjects did not wear nose clips. EBC samples were snap-frozen in liquid nitrogen before being stored at −80°C in polypropylene vial tubes until analytical determinations. EBC from smokers was collected at least 8 hours after refraining from cigarette smoking.

In this study we ascertained the contamination of saliva in EBC through the colorimetric detection of α-amylase (Infinity amylase reagent; Sigma, St. Louis, MO).

Aldehyde Determinations

Chemicals and reagents. MDA tetrabutylammonium salt (purity, 98%) was purchased from Fluka (Sigma-Aldrich, Milan, Italy). *n*-Alkanal standards (*n*-hexanal, *n*-heptanal, and *n*-nonanal; purity, more than 95%) and 2,4-dinitrophenylhydrazine (purity, 97%) were purchased from Sigma-Aldrich. All these standards were used without further purification. 4-Hydroxynonenal (purity, more than 98%) and 4-hydroxyhexenal (purity, 98%) dissolved in ethanol (5 mg/500 μl) were obtained from Cayman Chemicals (Ann Arbor, MI). High-performance liquid chromatography-grade water, methanol, and acetonitrile were from LabScan (Dublin, Ireland). Analytical-grade acetic acid and formic acid were from Fluka.

Aldehyde measurements. MDA, α,β-unsaturated aldehydes, and saturated aldehydes were determined, after derivatization with 2,4-dinitrophenylhydrazine, by liquid chromatography–tandem mass spectrometry (API 365; PerkinElmer Sciex, Thornhill, Canada). Briefly, ionization of the analytes was obtained by atmospheric pressure chemical ionization in positive-ion mode for MDA, and in negative-ion mode for 4-hydroxynonenal and 4-hydroxyhexenal and saturated aldehydes. Dinitrophenylhydrazone derivatives were separated on a Supelcosil C_{18} DB column (75 × 4.6 mm i.d., 3 μm; Supelco, Bellefonte, PA), using variable proportions of 20 mM aqueous acetic acid and methanol.

Methodological Issues in the Standardization of Aldehyde EBC Analysis

EBC was collected from a mixed group of smoking and nonsmoking subjects to verify a possible influence of methodological issues related to EBC collection (20) on aldehyde levels. Studies 1–5 were conducted with the above-described condensing device.

Study 1: Assessment of the influence of expiratory flows on EBC aldehyde levels. EBC from six healthy subjects was collected at four constant expiratory flows (200, 150, 100, and 50 ml/second). Different expiratory resistances were created with a restrictor set (HTF 5019X; Sievers Instruments, Boulder, CO) currently used for exhaled nitric oxide measurement at multiple exhaled flow rates (21). An ultrasonic flow sensor (ndd Medizintechnik, Zurich, Switzerland) was inserted between the mouth of the subject and the condenser. The flow meter was connected to a computer display provided with special software (Ecomedics, Duernten, Switzerland) for visual control of the expiratory flow. The ultrasonic flow meter is particularly well suited for this purpose, as the exhaled air is not trapped or filtered.

Study 2: Influence of the exhaled air volume, and of the amount of produced EBC on aldehyde levels. The volume of air exhaled during the EBC maneuver was registered with a special volume counter (SpiroPro; Jaeger, Hoechberg, Germany).

Study 3: Effect of EBC collection time on aldehyde levels. Ten subjects were asked to collect EBC in 10 minutes and in 20 minutes.

Study 4: Day-to-day reproducibility. Eight subjects were asked to collect EBC on two different, usually subsequent, days.

Study 5: Effect of acute smoking on EBC aldehyde levels. Seven smokers were asked to collect EBC before and immediately after having smoked one cigarette.

Study 6: Comparison of two condensing devices on EBC aldehyde levels. EBC was collected from 10 subjects, using both the above-described tubing condenser and a commercially available condenser (EcoScreen; Jaeger) as described previously (22). Briefly, subjects were asked to breath tidally for 10 or 20 minutes without a nose clip in place, through a two-way nonrebreathing valve by which inspiratory and expiratory air are separated, and saliva is trapped.

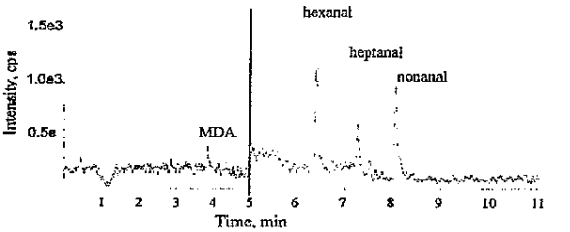

Figure 1. Chromatogram of the different classes of aldehydes measured in a sample of EBC: Malondialdehyde (*MDA*) = 16.3 nmol/L; *hexanal* = 25.4 nmol/L; *heptanal* = 17.1 nmol/L; *nonanal* = 20.2 nmol/L; *cps* = counts per second.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism, version 3.0 (GraphPad Software, San Diego, CA). Data were expressed as means ± standard error of the mean. Comparisons between three groups were evaluated by one-way analysis of variance followed by Mann–Whitney U test for comparison between two groups. Correlations between variables were evaluated by Spearman test. A p value of less than 0.05 indicated statistical significance. The Bland–Altman test (23) was used to evaluate the agreement between the methods of measurement and to evaluate the reproducibility.

RESULTS

EBC was collected without discomfort and none of the EBC samples showed detectable levels of α-amylase activity. A typical chromatogram of the aldehydes in EBC from a control subject is shown in Figure 1.

Standardization of Aldehyde Collection and Analysis by EBC

Study 1: Assessment of the influence of expiratory flows on EBC aldehyde levels. The concentrations of MDA in EBC collected at flow rates of 200, 150, 100, and 50 ml/second were 19.5 ± 3.2, 17.5 ± 1.2, 17.4 ± 0.1, and 17.8 ± 1.6 nmol/L, respectively. No statistically significant differences were observed among MDA values obtained at the different flow rates and no correlation was found between exhaled flow rates and MDA levels. No influence of exhaled flows on saturated aldehyde levels was observed (data not shown).

Study 2: Influence of the exhaled air volume, and of the amount of produced EBC on aldehyde levels. The mean values of exhaled air collected during the EBC maneuver in 10 and 20 minutes were 120.6 ± 38 and 224.1 ± 79.8 L, respectively, and no correlation was found with the relative aldehyde levels. The mean volume of EBC during 10 and 20 minutes of tidal breathing was

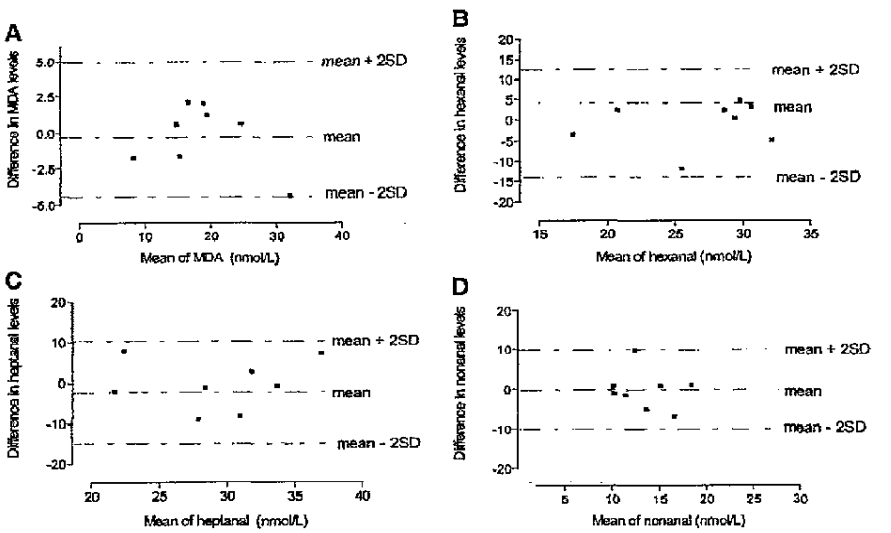

Figure 2. Day-to-day reproducibility of aldehyde measurements. Data were obtained from nonsmoking volunteers and smokers on (*A*) MDA, (*B*) hexanal, (*C*) heptanal, and (*D*) nonanal.

1.22 ± 0.1 and 2.1 ± 0.1 ml, respectively, and no correlation was found with the relative aldehyde concentrations.

Study 3: Effect of EBC collection time on aldehyde levels. No differences were observed in aldehyde levels in EBC collected in 10 and 20 minutes: MDA, 16.4 ± 2.4 versus 17.1 ± 1.7 nmol/L; hexanal, 28.9 ± 2.8 versus 29.3 ± 2.6 nmol/L; heptanal, 15.9 ± 1.2 versus 18.0 ± 2.0 nmol/L; nonanal, 19.0 ± 1.8 versus 17.9 ± 3.5 nmol/L.

Study 4: Day-to-day reproducibility. The mean coefficient of variation was 8.2% for MDA, 12.9% for hexanal, 14.3% for heptanal, and 10.3% for nonanal. The reproducibility was confirmed by the Bland–Altman test (Figure 2).

Study 5: Effect of acute smoking on EBC aldehyde levels. No difference was observed in aldehyde levels before and after smoking one cigarette: MDA, 31.0 ± 5.2 versus 36.6 ± 8.8 nmol/L; hexanal, 62.9 ± 5.7 versus 68.6 ± 7.2 nmol/L; heptanal, 32.5 ± 2.8 versus 33.3 ± 1.4 nmol/L; nonanal, 14.4 ± 3.1 versus 16.0 ± 2.4 nmol/L, respectively.

Study 6: Comparison of the effect of two condensing devices on EBC aldehyde levels. The coefficient of correlation was $r = 0.9$, $p < 0.01$ for MDA, $r = 0.9$, $p < 0.01$ for hexanal, $r = 0.9$, $p < 0.01$ for heptanal, $r = 0.8$, $p < 0.05$ for nonanal. The limit of agreement was confirmed by the Bland–Altman test (Figure 3).

Aldehyde Levels in EBC

MDA, hexanal, heptanal, and nonanal were detectable in the EBC of all subjects and their levels are shown in Table 1. Hydroxylated aldehydes were measurable only in a few samples.

MDA levels showed differences within groups ($p < 0.0001$). MDA was increased in the EBC of patients with COPD compared with smoking control subjects ($p = 0.0007$) and nonsmoking control subjects ($p < 0.0001$). In smoking control subjects, MDA levels were increased compared with nonsmoking control subjects ($p < 0.0001$) (Figure 4).

Hexanal showed differences within groups ($p = 0.002$). Hexanal was increased in the EBC of patients with COPD compared with nonsmoking control subjects ($p = 0.004$) but not compared with smoking control subjects ($p = 0.1$). In smoking control subjects, hexanal levels were increased compared with nonsmoking control subjects ($p < 0.006$).

Heptanal showed differences within groups ($p < 0.0001$). Heptanal was increased in the EBC of patients with COPD compared with nonsmoking control subjects ($p = 0.002$) but not compared with smoking control subjects. In smoking control subjects, heptanal levels were increased compared with nonsmoking control subjects ($p = 0.002$).

Nonanal showed no differences within groups ($p > 0.05$).

No difference in EBC aldehyde levels was observed between current and ex-smoking patients with COPD: MDA, 62.3 ± 4.7 versus 54.2 ± 2.4 nmol/L; hexanal, 58.7 ± 2.4 versus 66.3 ± 6.9 nmol/L; heptanal, 32.5 ± 7.7 versus 23.1 ± 3.6 nmol/L; nonanal, 19.1 ± 2.8 versus 21.1 ± 2.5 nmol/L, respectively.

Cigarette consumption was slightly lower in smoking control subjects than in smoking patients with COPD, although the difference was not statistically significant ($p = 0.07$). No correlation was found between aldehyde levels and smoking history. The ex-smokers with COPD still had higher MDA levels than did smoking control subjects ($p = 0.002$).

DISCUSSION

In this study, we verified that different aldehydes can be measured in EBC by liquid chromatography-tandem mass spectrometry and that analytical values are reproducible and unaffected by sampling procedures. MDA, hexanal, and heptanal, but not nonanal, were increased in patients with COPD in comparison to nonsmoking control subjects. Only MDA levels were increased in patients with COPD compared with smoking control subjects.

Oxidative stress is increased in patients with COPD and ROS contribute to its pathophysiology (1, 2). Three major sources of ROS have been identified in COPD: (*1*) cigarette smoking, generating free radicals and quinones undergoing redox cycling (1); (*2*) neutrophil activation characterizing the inflammatory reaction (1); and (*3*) reoxygenation injury, which has also been associated with COPD (24). ROS have several deleterious effects in COPD, including activation of the transcription factor nuclear factor-κB, switching on of genes encoding proinflammatory cytokines, such as tumor necrosis factor-α and interleukin-8. Oxidative damage to antiproteases such as α_1-antitrypsin and the secretory leukoprotease inhibitor is another effect of oxidative stress enhancing proteolytic injury in COPD (25).

Previous studies showed high levels of lipoperoxidation prod-

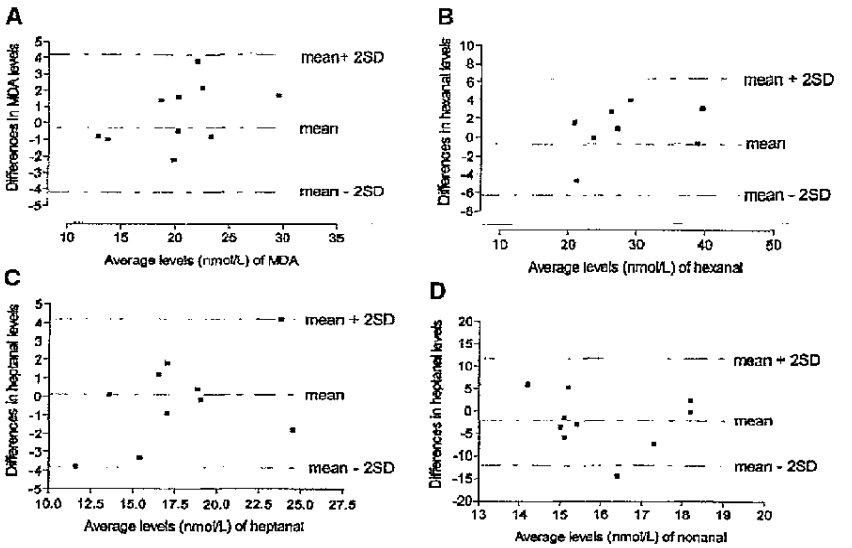

Figure 3. Comparison of the effect of two condensing devices (Tygon condenser versus Jaeger condenser) on EBC aldehyde levels. Data were obtained from nonsmoking volunteers and smokers on (*A*) *MDA*, (*B*) *hexanal*, (*C*) *heptanal*, and (*D*) *nonanal*.

ucts in blood and lungs of smokers and patients with COPD (26–28). Exhaled gases, such as nitric oxide and carbon monoxide, have been proposed as noninvasive markers of inflammation and oxidative stress. Exhaled nitric oxide is increased in asthma (5) and in COPD during exacerbations (29), whereas conflicting results have been reported for patients with clinically stable COPD (30). This could derive from the fact that airway nitric oxide is consumed by its reaction with ROS, yielding the powerful oxidant peroxynitrite, which is involved in the pathophysiology of COPD (31). High levels of carbon monoxide can be measured in exhaled air of patients with COPD (32). However, carbon monoxide is also markedly increased by cigarette smoking. Oxidative stress in smokers and in patients with COPD is also indicated by markers of lipoperoxidation. Ethane and pentane are increased in exhaled air of smokers and patients with COPD as volatile breakdown products of polyunsaturated fatty acids (33, 34). High levels of 8-isoprostane, a prostaglandin-$F_{2\alpha}$ isomer that is formed *in vivo* by peroxidation of arachidonic acid, have also been reported in EBC from patients with COPD (8).

In the present study, we measured aldehydes as biomarkers of the attack of ROS on unsaturated lipids in membranes. Aldehyde generation aggravates the effects of ROS and underlying changes in cells and tissues associated with oxidative stress (35). MDA and saturated aldehydes, but not nonanal, were increased in EBC from patients with COPD as compared with nonsmoking control subjects and in EBC from smoking as compared with nonsmoking control subjects. No differences were observed between smoking and ex-smoking patients with COPD as to EBC aldehyde concentrations, although current smokers exhibited a tendency toward higher levels. This fits nicely with the observation of Nowak and coworkers (14), who showed slightly higher thiobarbituric acid-reactive substances in EBC from still-smoking patients with COPD than in ex-smokers. Thus, smoking cessation per se does not eliminate the oxidative stress associated with COPD, and aldehyde levels in EBC can be used to monitor subsequent damage to cell membranes.

The fact that only MDA distinguished patients with COPD from smoking control subjects is consistent with the results of Montuschi and coworkers (8), who showed a gradient of 8-isoprostane concentrations in EBC from patients with COPD to smoking subjects and nonsmoking control subjects, respectively. Our data are also in agreement with those of Rahman and coworkers (36), who measured higher levels of aldehyde adducts in lung epithelial cells of subjects with COPD compared with

TABLE 1. ALDEHYDES IN EXHALED BREATH CONDENSATE FROM NONSMOKING CONTROL SUBJECTS, SMOKING CONTROL SUBJECTS, AND PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

		Aldehyde Concentration, nmol/L		
	LOD (nmol/L)	Nonsmoking Control Subjects (n = 20)	Smoking Control Subjects (n = 12)	Patients with COPD (n = 20)
Malondialdehyde	1.07	12.1 ± 1.8	35.6 ± 4.0*	57.2 ± 2.4*†
Hexanal	1.07	17.7 ± 5.0	64.9 ± 3.3*	63.5 ± 4.4*
Heptanal	0.34	14.2 ± 3.5	29.6 ± 2.3*	26.6 ± 3.9*
Nonanal	0.31	18.7 ± 0.9	14.8 ± 2.4	20.4 ± 1.8

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; LOD = limit of detection (nmol/L).
Data are expressed as mean concentrations ± SEM.
* $p < 0.05$, versus nonsmoking control subjects.
† $p < 0.05$, versus smoking control subjects.

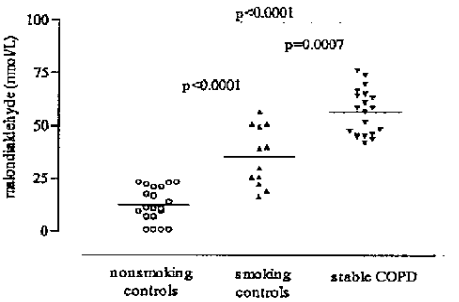

Figure 4. Comparison of malondialdehyde levels in exhaled breath condensate from nonsmoking control subjects, smoking control subjects, and patients with stable, moderate to severe *COPD*.

smokers without COPD. On the other hand, Maestrelli and coworkers (37) found that lung tissue expression of heme oxygenase, an enzyme whose induction protects from oxidant-mediated cellular injury, is increased in the lungs of smokers, but does not distinguish healthy smokers from patients with COPD. From our results, oxidative stress seems to be more pronounced in smokers and ex-smokers who developed COPD compared with smokers who did not. In particular, there could be a threshold for MDA levels separating membrane damage resulting from oxidative damage due to smoking alone, from that caused by biochemical reactions associated with tissue changes in COPD.

Nonanal levels in COPD were not different from those of nonsmoking control subjects, nor were they increased in smoking compared with nonsmoking control subjects. The patterns of aldehyde subtypes from different groups did not overlap. Possible explanations may include differences in lipid composition of epithelial lining fluid (38), variability in scavenging capacity (39), complexity of target inflammatory/constitutive cells (40), variable effects of ROS on macromolecular targets at the cell and membrane levels (15), and increased epithelial permeability to plasma proteins forming adducts with aldehydes (41). Experiments aimed at characterizing the aldehyde patterns generated by different challenges to various cell lines *in vitro* are being conducted.

In all groups, α,β-unsaturated aldehydes were detectable only in a few EBC samples. These aldehydes are highly reactive and are either scavenged rapidly by thiol groups (15) or form aldehyde adducts with cell macromolecules. The former mechanism is thought to give rise to specific adducts that, at least in part, could be released and quantified in EBC; suitable methods of analysis are being developed in our laboratory for this purpose. The latter possibility is supported by a study by Rahman and coworkers (36) showing increased levels of 4-hydroxy-2-nonenal adducts in lung epithelial and endothelial cells as well as in neutrophils in COPD.

Although cigarette consumption was slightly lower in smoking control subjects than in smoking patients with COPD, smoking history does not explain the difference in aldehyde levels, as no correlation was found between aldehyde levels and pack-years. Moreover, COPD ex-smokers who had stopped smoking for at least 1 year still had MDA levels higher than those of smoking control subjects, thus suggesting that mechanisms other than smoking underlie the marked lipoperoxidation associated with the disease. Smoking control subjects and patients with COPD were not age matched ($p = 0.005$), although we are confident that age cannot affect aldehyde expression as EBC aldehyde levels did not correlate with age, in agreement with Frampton and coworkers (42) and Montuschi and coworkers (8), who were also unable to find any correlation between lipid peroxides and age.

In our study, we found no statistically significant correlation between EBC aldehyde levels and spirometric values, such a lack of correlation being in line with previous reports (9, 43). This could be related to the fact that spirometric values (FEV_1 and FVC) are measures of the caliber of the central (large) airways, whereas EBC may preferentially sample small airways during the popping open of closed respiratory bronchioles (11), where most of the COPD pathology takes place (44). However, this is just an hypothesis and we acknowledge that, at the current stage of research, there is no way to identify the anatomic sites from which EBC samples are taken. Further studies addressing this issue, as well as studies dealing with the mechanism of formation of EBC, are necessary. At this stage we are not proposing EBC as a screening tool for patients with moderate/severe COPD. The utility of EBC as a screening tool for oxidative stress and tissue injury in COPD should be addressed in future studies using asymptomatic smokers and patients with mild COPD that will be monitored for an extended period of time.

The levels of MDA we observed are similar to those reported by Lärstad and coworkers (45) in the EBC of subjects with asthma. MDA and saturated aldehydes were present in the same order of magnitude as in bronchoalveolar lavage (42) and induced sputum (our unpublished observation). Compared with the latter, EBC has the great advantage that it is simple to collect, totally noninvasive, and based on portable devices, and therefore it has the potential to be applicable in outpatient settings or even at home.

In the present study, we addressed methodological issues, to clarify their possible influence on EBC aldehyde measurements. The concentration of aldehydes was not related to sampling time and hence to the total volume of EBC, thus suggesting a constant production rate over variable sampling periods. Aldehyde levels in EBC showed acceptable day-to-day variations and were not influenced by the sampling device.

How EBC is generated and where it is sampled from are still a matter for speculation. One hypothesis is that airway opening dynamics and turbulence in the airways churn up droplets containing substances from the airway lining fluid. If so, a positive correlation between solute concentrations and exhaled flows could be expected. Our data showed a trend toward increased MDA levels at 200 ml/second (19.5 mmol/L) versus the other three flows tested (MDA range, 17.4–17.8 mmol/L), although the difference was not statistically significant, nor were we able to find any correlation between exhaled flows and EBC aldehyde levels. This observation is in line with that reported by Montuschi and coworkers (46), who did not find a correlation between EBC 8-isoprostane and exhaled flow rates, and with the studies of Carpenter and coworkers (47) and of Reinhold and coworkers (48), who showed that the volume of EBC is dependent on the ventilation volume per unit time, but this does not affect the EBC concentration of mediators. A possible explanation for the lack of correlation is that an increase in exhaled velocity can result in enhanced aerosolization of particles during forced exhalations (49). However, this was not the case in our study, as the volunteers were asked to breathe not forcedly, although at different expired flows. Furthermore, the observed variable changes in biomarkers argue against EBC analysis representing the increase in the number of aerosolized droplets recovered in the EBC. On the other hand, Schleiss and coworkers (50) reported a negative correlation between EBC hydrogen peroxide levels and exhaled flows.

In conclusion, in the present study we identified different classes of aldehydes in EBC, using liquid chromatography–tandem

mass spectrometry, and we verified that aldehyde measurements are unaffected by EBC sampling procedures. MDA, hexanal, and heptanal are increased in the EBC of patients with COPD in comparison with nonsmoking control subjects. EBC is a simple, noninvasive, and easily repeatable procedure for the evaluation of oxidative stress in airway diseases. The possibility of using a reference analytical technique for measuring biomarkers of oxidative stress in EBC could open the way to using these biomarkers to assess oxidative stress status in clinical practice and to predict the usefulness of antioxidant drugs.

Acknowledgment: The authors thank Brijesh Kumar and Olga Acampa for assistance during the course of this study.

References

1. Repine JE, Bast A, Lankhorst I. Oxidative stress in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1997;156:341–357.
2. MacNee W. Oxidative stress and lung inflammation in airways disease. *Eur J Pharmacol* 2001;429:195–207.
3. Ferreira IM, Hazari MS, Gutierrez C, Zamel N, Chapman KR. Exhaled nitric oxide and hydrogen peroxide in patients with chronic obstructive pulmonary disease: effects of inhaled beclomethasone. *Am J Respir Crit Care Med* 2001;164:1012–1015.
4. Barnes PJ. New treatment for COPD. *Nat Rev Drug Discov* 2002;1: 437–446.
5. Kharitonov SA, Barnes PJ. Exhaled markers of pulmonary disease. *Am J Respir Crit Care Med* 2001;163:1693–1722.
6. Mutlu GM, Garey KW, Robbins RA, Danziger LH, Rubinstein I. Collection and analysis of exhaled breath condensate in humans. *Am J Respir Crit Care Med* 2001;164:731–737.
7. Van Beurden WJ, Dekhuijzen PN, Harff GA, Smeenk FW. Variability of exhaled hydrogen peroxide in stable COPD patients and matched healthy controls. *Respiration* 2002;69:211–216.
8. Montuschi P, Collins JV, Ciabattoni G, Lazzeri N, Corradi M, Kharitonov SA, Barnes PJ. Exhaled 8-isoprostane as an *in vivo* biomarker of lung oxidative stress in patients with COPD and healthy smokers. *Am J Respir Crit Care Med* 2000;162:1175–1177.
9. Dekhuijzen PN, Aben KK, Dekker I, Aarts LP, Wielders PL, van Herwaarden CL, Bast A. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1996;154:813–816.
10. Corradi M, Montuschi P, Donnelly LE, Pesci A, Kharitonov SA, Barnes PJ. Increased nitrosothiols in exhaled breath condensate in inflammatory airway diseases. *Am J Respir Crit Care Med* 2001;163:854–858.
11. Hunt J. Exhaled breath condensate: an evolving tool for noninvasive evaluation of lung disease. *J Allergy Clin Immunol* 2002;110:28–34.
12. Liebler DC, Reed DJ. Free-radical defence and repair mechanisms. In: Wallance KB, editor. Free radical toxicology. New York: Publisher Press; 1999. p. 141–171.
13. Nowak D, Kalucka S, Bialasiewicz P, Krol M. Exhalation of H_2O_2 and thiobarbituric acid reactive substances (TBARs) by healthy subjects. *Free Radic Biol Med* 2001;30:178–186.
14. Nowak D, Kasielski M, Antczak A, Pietras T, Bialasiewicz P. Increased content of thiobarbituric acid-reactive substances and hydrogen peroxide in the expired breath condensate of patients with stable chronic obstructive pulmonary disease: no significant effect of cigarette smoking. *Respir Med* 1999;93:389–396.
15. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes. *Free Radic Biol Med* 1991;11:81–128.
16. Pryor WA, Das B, Church DF. The ozonation of unsaturated fatty acids: aldehydes and hydrogen peroxide as products and possible mediators of ozone toxicity. *Chem Res Toxicol* 1991;4:341–348.
17. Gelpi E. Biomedical and biochemical applications of liquid chromatography–mass spectrometry. *J Chromatogr A* 1995;703:59–80.
18. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop Summary. *Am J Respir Crit Care Med* 2001;163:1256–1276.
19. Ho LP, Innes JA, Greening AP. Nitrite levels in breath condensate of patients with cystic fibrosis is elevated in contrast to exhaled nitric oxide. *Thorax* 1998;53:680–684.
20. Montuschi P, Barnes PJ. Analysis of exhaled breath condensate for monitoring airway inflammation. *Trends Pharmacol Sci* 2002;23:232–237.
21. Lehtimäki L, Turjanmaa V, Kankaanranta H, Saarelainen S, Hahtola P, Moilanen E. Increased bronchial nitric oxide production in patients with asthma measured with a novel method of different exhalation flow rates. *Ann Med* 2000;32:417–423.
22. Corradi M, Alinovi R, Goldoni M, Vettori M, Folesani G, Mozzoni P, Cavazzini S, Bergamaschi E, Rossi L, Mutti A. Biomarkers of oxidative stress after controlled human exposure to ozone. *Toxicol Lett* 2002; 134:219–225.
23. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307–310.
24. Pinamonti S, Leis M, Barbieri A, Leoni D, Muzzoli M, Sostero S, Chicca MC, Carrieri A, Ravenna F, Fabbri LM, Ciaccia A. Detection of xanthine oxidase activity products by EPR and HPLC in bronchoalveolar lavage fluid from patients with chronic obstructive pulmonary disease. *Free Radic Biol Med* 1991;25:771–779.
25. Barnes PJ. Mechanisms in COPD: differences from asthma. *Chest* 2000; 117:10S–14S.
26. Rahman I, Morrison D, Donaldson K, MacNee W. Systemic oxidative stress in asthma, COPD, and smokers. *Am J Respir Crit Care Med* 1996;154:1055–1060.
27. Morrison D, Rahman I, Lannan S, MacNee W. Epithelial permeability, inflammation, and oxidant stress in the airspaces of smokers. *Am J Respir Crit Care Med* 1999;159:473–479.
28. Morrow JD, Frei B, Longmire AW, Gaziano JM, Lynch SM, Shyr Y, Strauss WE, Oates JA, Roberts LJ II. Increase in circulating products of lipid peroxidation (F_2-isoprostanes) in smokers: smoking as a cause of oxidative damage. *N Engl J Med* 1995;332:1198–1203.
29. Maziak W, Loukides S, Culpitt S, Sullivan P, Kharitonov SA, Barnes PJ. Exhaled nitric oxide in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;157:998–1002.
30. Papi A, Romagnoli M, Baraldo S, Braccioni F, Guzzinati I, Saetta M, Ciaccia A, Fabbri LM. Partial reversibility of airflow limitation and increased exhaled NO and sputum eosinophilia in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;162:1773–1777.
31. Ichinose M, Sugiura H, Yamagata S, Koarai A, Shirato K. Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. *Am J Respir Crit Care Med* 2000;162:701–706.
32. Montuschi P, Kharitonov SA, Barnes PJ. Exhaled carbon monoxide and nitric oxide in COPD. *Chest* 2001;120:496–501.
33. Paredi P, Kharitonov SA, Leak D, Ward S, Cramer D, Barnes PJ. Exhaled ethane, a marker of lipid peroxidation, is elevated in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;162:369–373.
34. Habib MP, Clements NC, Garewal HS. Cigarette smoking and ethane exhalation in humans. *Am J Respir Crit Care Med* 1995;151:1368–1372.
35. Gutteridge JML. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin Chem* 1995;41:1819–1828.
36. Rahman I, van Schadewijk AAM, Crowther AJL, Hiemstra PS, Stolk J, MacNee W, De Boer WI. 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2002;166: 490–495.
37. Maestrelli P, El Messlemani AH, De Fina O, Nowicki Y, Saetta M, Mapp C, Fabbri LM. Increased expression of heme oxygenase (HO)-1 in alveolar spaces and HO-2 in alveolar walls of smokers. *Am J Respir Crit Care Med* 2001;164:1508–1513.
38. Honda Y, Tsunematsu K, Suzuki A, Akino T. Changes in phospholipids in bronchoalveolar lavage fluid of patients with interstitial lung diseases. *Lung* 1988;166:293–301.
39. Taylor JC, Madison R, Kosinska D. Is antioxidant deficiency related to chronic obstructive pulmonary disease? *Am Rev Respir Dis* 1986;134: 285–289.
40. Saetta M, Turato G, Maestrelli P, Mapp CE, Fabbri LM. Cellular and structural bases of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;163:1304–1309.
41. Hamilton RF Jr, Hazbun ME, Jumper CA, Eschenbacher WL, Holian A. 4-Hydroxynonenal mimics ozone-induced modulation of macrophage function ex vivo. *Am J Respir Cell Mol Biol* 1996;15:275–282.
42. Frampton MW, Pryor WA, Cueto R, Cox C, Morrow PE, Utell MJ. Aldehydes (nonanal and hexanal) in rat and human bronchoalveolar lavage fluid after ozone exposure. *Res Rep Health Eff Inst* 1999;90:1–15.
43. Pratico D, Basili S, Vieri M, Cordova C, Violi F, Fitzgerald GA. Chronic obstructive pulmonary disease is associated with an increase in urinary levels of isoprostane $F_{2\alpha}$-111, an index of oxidant stress. *Am J Respir Crit Care Med* 1998;158:1709–1714.
44. Cosio Piqueras MG, Cosio MG. Disease of the airways in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 2001;34:41s–49s.
45. Lärstad M, Ljungkvist G, Olin AC, Toren K. Determination of malondial-

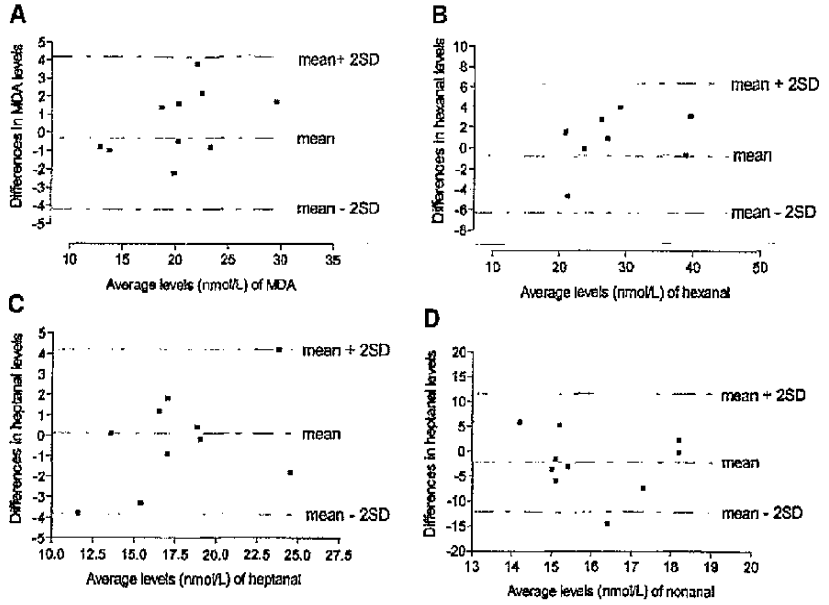

Figure 3. Comparison of the effect of two condensing devices (Tygon condenser versus Jaeger condenser) on EBC aldehyde levels. Data were obtained from nonsmoking volunteers and smokers on (*A*) *MDA*, (*B*) *hexanal*, (*C*) *heptanal*, and (*D*) *nonanal*.

ucts in blood and lungs of smokers and patients with COPD (26–28). Exhaled gases, such as nitric oxide and carbon monoxide, have been proposed as noninvasive markers of inflammation and oxidative stress. Exhaled nitric oxide is increased in asthma (5) and in COPD during exacerbations (29), whereas conflicting results have been reported for patients with clinically stable COPD (30). This could derive from the fact that airway nitric oxide is consumed by its reaction with ROS, yielding the powerful oxidant peroxynitrite, which is involved in the pathophysiology of COPD (31). High levels of carbon monoxide can be measured in exhaled air of patients with COPD (32). However, carbon monoxide is also markedly increased by cigarette smoking. Oxidative stress in smokers and in patients with COPD is also indicated by markers of lipoperoxidation. Ethane and pentane are increased in exhaled air of smokers and patients with COPD as volatile breakdown products of polyunsaturated fatty acids (33, 34). High levels of 8-isoprostane, a prostaglandin-$F_{2\alpha}$ isomer that is formed *in vivo* by peroxidation of arachidonic acid, have also been reported in EBC from patients with COPD (8).

In the present study, we measured aldehydes as biomarkers of the attack of ROS on unsaturated lipids in membranes. Aldehyde generation aggravates the effects of ROS and underlying changes in cells and tissues associated with oxidative stress (35). MDA and saturated aldehydes, but not nonanal, were increased in EBC from patients with COPD as compared with nonsmoking control subjects and in EBC from smoking as compared with nonsmoking control subjects. No differences were observed between smoking and ex-smoking patients with COPD as to EBC aldehyde concentrations, although current smokers exhibited a tendency toward higher levels. This fits nicely with the observation of Nowak and coworkers (14), who showed slightly higher thiobarbituric acid-reactive substances in EBC from still-smoking patients with COPD than in ex-smokers. Thus, smoking cessation per se does not eliminate the oxidative stress associated with COPD, and aldehyde levels in EBC can be used to monitor subsequent damage to cell membranes.

The fact that only MDA distinguished patients with COPD from smoking control subjects is consistent with the results of Montuschi and coworkers (8), who showed a gradient of 8-isoprostane concentrations in EBC from patients with COPD to smoking subjects and nonsmoking control subjects, respectively. Our data are also in agreement with those of Rahman and coworkers (36), who measured higher levels of aldehyde adducts in lung epithelial cells of subjects with COPD compared with

TABLE 1. ALDEHYDES IN EXHALED BREATH CONDENSATE FROM NONSMOKING CONTROL SUBJECTS, SMOKING CONTROL SUBJECTS, AND PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

		Aldehyde Concentration, nmol/L		
	LOD (*nmol/L*)	Nonsmoking Control Subjects (*n = 20*)	Smoking Control Subjects (*n = 12*)	Patients with COPD (*n = 20*)
Malondialdehyde	1.07	12.1 ± 1.8	35.6 ± 4.0*	57.2 ± 2.4*†
Hexanal	1.07	17.7 ± 5.0	64.9 ± 3.3*	63.5 ± 4.4*
Heptanal	0.34	14.2 ± 3.5	29.6 ± 2.3*	26.6 ± 3.9*
Nonanal	0.31	18.7 ± 0.9	14.8 ± 2.4	20.4 ± 1.8

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; LOD = limit of detection (nmol/L).
Data are expressed as mean concentrations ± SEM.
* $p < 0.05$, versus nonsmoking control subjects.
† $p < 0.05$, versus smoking control subjects.